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Evidence for enhanced late-stage larval quality, not survival, through maternal carry-over effects in a space monopolizing barnacle

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ABSTRACT

Understanding the effects of maternal and pelagic resource allocation on larval traits is essential to better understand population dynamics of marine benthic invertebrates. We tested how different levels of food supply to adult barnacles and their feeding larvae (nauplii) might alter survival to the settling cyprid larval stage and cyprid quality. Median development time did not vary, except when both parents and larvae were given a low food supply, which delayed the time to metamorphosis by over 40%. Survival to the cyprid stage was only affected by larval feeding, which doubled in better-fed nauplii. In contrast, cyprid size showed a more complex response, prone to additive effects of maternal and larval provisioning. Moreover, the resulting size-range observed for experimental cyprids (spanning over 70% of the minimum cyprid size) mirrored the variation found in the coastal plankton, suggesting that food supply may exert similar effects in nature. Given that barnacles nearly saturate available habitat under favorable conditions, maternal allocation resulting in enhanced late-stage larval quality may be adaptive since competition for available settling space is likely intense. On the other side, severe resource limitation through embryogenesis and larval development may impose delayed metamorphosis and thus enhanced potential for transport and the colonization of marginal habitats, where intraspecific competition may be lower and larval quality less critical.

KEYWORDS: *Chthamalus bisinuatus*; supply side ecology; sessile invertebrates; South Atlantic; tropical coast

INTRODUCTION

Recruitment is an important process controlling population dynamics, but key processes determining input to mature stocks are still not well understood. In marine invertebrates with complex life cycles, the supply of final-stage larvae to benthic habitat has long been considered a critical factor controlling recruitment success (e.g. Jenkins et al., 2008; Pineda et al., 2009). However, larval supply can be largely decoupled from settlement rate (Miron et al., 1995; Moreira et al., 2007), and settlers of the same larval pool can show quite different performance once established in the benthic environment (growth and survival rates combined; Jarrett, 2003; McQuaid & Phillips, 2006), suggesting that larval physiological conditions, i.e. larval quality, may play a very important role. Indeed, an increasing number of studies using different proxies of larval quality, such as size and organic content, have shown that good-quality late-stage larvae of barnacles and crabs develop into juveniles that grow faster and survive longer (e.g. Jarret, 2003; Emlet & Sadro, 2006; Giménez, 2010). Effects of larval quality may endure until adulthood, as shown for barnacles (Torres et al., 2016) and for colonial bryozoans (Marshall et al., 2003). In the case of bryozoans, colonies originating from large larvae reached maturity earlier and produced more embryos compared to colonies originating from smaller larvae (Marshall et al., 2003). Converging evidence from experimental work on different marine invertebrate groups has therefore led to a broad recognition of the importance of larval quality on recruitment strength and population dynamics (Burgess & Marshall, 2011; Van Allen & Rudolf, 2013).

Most marine invertebrates produce feeding or non-feeding larval stages that remain in the plankton for periods of several days to a few weeks (Shanks et al., 2003), during which pelagic transport may take larvae to distant habitat patches where population density and resource availability may be quite different from those at the natal location. How this uncertainty has shaped patterns of maternal provisioning to offspring, and particularly how it affects the number of potential settlers and their quality, is still an open question.

Patterns of maternal provisioning can be variable between the production of lecithotrophic and planktotrophic larvae. Lecithotrophic larvae rely solely on maternal provisioning during their often very short pelagic lifetime. In species producing such non-feeding larvae, environmental variables reflecting benthic habitat quality may shape patterns of maternal resource manipulation during embryogenesis (i.e. maternal effects) that modulate offspring number and quality, as well as their dispersal potential (Marshall & Keough, 2006; Marshall & Uller, 2007). The case of planktotrophic larvae is more complex because maternal provisioning acts in addition to pelagic feeding to improve general larval performance (e.g. Steer et al., 2004; Gagliano & McCormick, 2007). This may be achieved through different mechanisms, such as reducing early offspring mortality through the allocation of essential precursors for basic physiological processes (e.g. Salze et al., 2005; Freuchet et al., 2015), or by inducing plasticity of different functional larval traits (Hart, 1995; George, 1999; McAlister, 2007; Reitzel & Heylan, 2007). Combined effects of maternal and pelagic resource allocation in feeding larvae

may therefore modulate two key determinants of recruitment – the supply and quality of potential settlers. The relevance of such determinants would depend on the strength of density-dependent processes controlling adult benthic populations. For habitat patches where population density is well below carrying-capacity (i.e. recruitment limited sites –Doherty & Fowler, 1994) increased planktonic larval survival rates leading to greater larval supply may be particularly advantageous to mothers, enhancing overall fitness. At sites of high population density where competition for space and food resources is likely intense (i.e. habitat limited – Holbrook et al., 2000), enhanced quality of settling larvae may be critical.

The species used in this study, the intertidal barnacle *Chthamalus bisinuatus* (Pilsbry), is the most common cirripede species along the subtropical Southeastern Atlantic, dominating the upper part of the eulittoral zone of rocky shores, where they frequently cover 80% or more of available substrates (Bueno et al., 2010). These filter feeding barnacles reproduce through cross fertilization - although some cases of self-fertilization have been reported for this genus (Barnes & Crisp, 1956; Anderson, 1994) - and the fertilized eggs are carried in the mantle cavity until the nauplius stage I is fully developed. During embryogenesis, and in the absence of chronic thermal stress, reserve lipids available to mothers are proportionally transferred to their embryos (Freuchet et al., 2015). Once in the plankton, nauplii feed and molt through 6 different stages until they metamorphose into the late non-feeding larval phase, the cyprid. Time-series analyses indicated a pelagic larval duration of 11-12 days in *C. bisinuatus* during summer (Barbosa et al., 2016), but this period may depend on a number of factors, e.g. temperature and food availability, as observed for other chthamalids (Egan & Anderson, 1989; Burrows et al., 1999; Yan & Chan, 2001). The energy accumulated until the cyprid phase will directly influence the exploration capacity to find an adequate settlement surface and the success of metamorphosis into the first juvenile stage (e.g. Jarrett, 2003; Tremblay et al., 2007).

We examined here how trophic resources supplied to parental barnacles and pelagic larvae modulate proxies of three critical recruitment components: *cyprid yield*, as an outcome of larval survival to the competent stage, and a surrogate of the supply of potential settlers; *median development time*, as a metric of pelagic larval duration and dispersal potential; and *cyprid size*, as a proxy of larval quality at the competent stage. This size-to-quality approximation relies on the assumption that larger cyprids metamorphose to larger juveniles, which in turn experience higher growth rates (e.g. Emlet & Sadro, 2006), likely providing a competitive advantage over other recruits and a faster development to a size refuge from predation or bulldozing by limpets (Gosselin & Qian, 1997). Also, this proxy is backed by the allometric scaling of metabolic rate, which predicts a relatively more efficient use of resources as size increases, and explains the generally better performance observed for larger recruits of a wide array of marine invertebrates (Pettersen et al., 2015). The potential for variation in these three larval traits would depend on whether they respond to single or

combined manipulations of maternal and pelagic resource allocation. Effects on traits responding to both resources could potentially be more extensive, especially if maternal and pelagic resources act independently and in the same direction (additive effects). We thus anticipated extended effects through maternal resource allocation (latent effects) combined with pelagic resource allocation, as observed for other marine invertebrate larvae (e.g in echinoderms; George, 1996; McAlister & Moran, 2013). We tested whether maternal and pelagic allocation contributes to traits affecting larval quality, i.e. cyprid size and traits associated with overall larval supply, i.e. survival rate and development time. Development time was assumed to contribute to larval supply since additional time in the plankton increases the risk of larval mortality through predation or transport away from suitable habitat. Carry-over effects on larval supply would support selection of trait responses under recruitment limitation, while such effects on larval quality would indicate selection of responses under habitat limitation.

METHODS

Experimental design - The effects of maternal and larval energy provisioning on the larval traits of *Chthamalus bisinuatus* were assessed through manipulation of adult and larval food supply in a factorial experiment [two levels of food supply; high (HF) and low (LF) for each life-stage] under constant room temperature of 21°C, corresponding to air temperature at the time of sampling (see below). Food supply consisted of an even mixture (i.e. equal cell volumes) of three microalgal species cultured in the laboratory, the flagellates *Tetraselmis* sp. and *Isochrysis* sp. and the diatom *Thalassiosira* sp. These species are common in the study region and are commonly used to rear marine invertebrate larvae (Stone, 1989; Egan & Anderson, 1989; Burrows et al., 1999; Helm et al., 2004). This mixture provided all essential fatty acids for larval growth (Ohse et al., 2015) and was delivered to both adults and larvae, at concentrations of either 3 X 10^3 cells.ml^{-1} (HF) or 1 X 10^3 cells.ml^{-1} (LF). Such concentrations were selected based on natural phytoplankton concentrations in the São Sebastião Channel (unpublished data).

Adult barnacles (mean opercular length: 2.3 mm ± 0.7 mm SD) were obtained by extracting 40 rock chips with dense cover from a 100 m stretch of a rocky coastline at Calhetas Head (23° 49' 54'' S; 45° 31' 18'' W), São Paulo State, Brazil, on July 16th 2014. Rock chips hosting a mean barnacle cover of 40.13% ± 3.96% (SD) were evenly and randomly assigned to two tanks (one for HF and one for LF treatments), ensuring that similar amount of adults were placed in each tank (54 x 40 x 20 cm). Adult barnacles were subjected to experimental diets for 20 days; a period that exceeds the interval of consecutive larval release events (12-13d; Kasten & Flores, 2013) and therefore should comprise the whole oogenic cycle, as in the tropical congener *Chthamalus malayensis* (i.e. 10 days, Yan et al., 2006). During this period, rock chips were submerged in filtered sea water (3 µm) containing the respective feeding medium twice a day for 1h, so as to simulate the semidiurnal tidal regime that prevails in the

area. Owing to logistical limitations, we could not replicate tanks within levels of adult food supply. However, a previous study on the same species, sampled from the same area and manipulated in the same laboratory space, showed that (i) the quantity of food supplied to adult barnacles proportionately translates to the quantity of lipid reserves in early larvae, and that (ii) larval survival did not differ among replicate aquaria for the combination of experimental treatments (adult food supply X thermal stress; Freuchet et al., 2015). As on that occasion, we carefully monitored temperature, light conditions and ventilation within the laboratory and kept them as constant as possible, which should have minimized the chances of any eventual environmental artifacts. Therefore, we are confident that the effects we report for maternal food supply are an outcome of maternal transference of resources, as previously observed.

After this manipulation, the nauplii released from experimental adult stocks were sampled by filtering the water through a 100 µm mesh. These larvae were then transferred to 500 ml glass beakers filled with 1 µm filtered seawater, aiming for a density of 0.5 nauplii.ml^{-1} (Moyse, 1960). A total of 12 beakers were prepared (n = 3 for each of the four treatment combinations, i.e. HF adult and HF larvae, LF adult and LF larvae, HF adult and LF larvae, LF adult and HF larvae). Beakers were randomly interspersed over the bench space to avoid any influence of environmental gradients within the laboratory. Every other day, nauplii were fed and 90% of the water volume in the beakers was renewed. After 10 days, beakers were carefully checked under a stereomicroscope (6-10X) for the presence of cyprids (either swimming or settled on the bottom or sides of beakers), which were removed from cultures, counted and preserved (70% ethanol) in separate vials. After the first appearance of cyprids, beakers were inspected every other day and the experiment terminated when no new cyprids were observed for a period of 10 days in any given beaker.

Response variables - Three different larval traits were separately quantified for each replicate beaker in each treatment combination. Cyprid yield (CY) was estimated as the percentage of nauplii that successfully survived to the cyprid stage. Median development time (MDT) was measured as the time (in days) by which 50% of all cyprids were obtained. Cyprid size (CS) was measured as the lateral carapace area (µm^2), from magnified images (63X) using the software ImageJ (Schneider et al., 2012). Linear cyprid metrics were inadequate because of substantial variation in carapace shape. In order to assess how results may actually translate to natural conditions, we compared the range of laboratory-reared cyprid sizes across treatments to the estimated range of near shore cyprids along the São Sebastião Channel. Plankton tows (150 µm) were taken from January 21st to March 28th 2013 at three different sites separated by a few kilometers: Praia do Segredo (23°49'44.06" S, 45°25'21.06" W), Saco Grande (23°49'55.23" S, 45°25'43.57" W) and Itassucê (23°49'56.77" S, 45°26'33.42" W). The general morphology and size were used to identify cyprids in samples. Several different barnacle species are found in the region, including balanids (mostly *Megabalanus* spp and *Balanus* spp), tetraclitids

(*Tetraclita stalactifera* [Lamarck]) and chthamalids. The cyprids observed in samples for the first two are much larger and slender (higher length/height ratios) compared to chthamlid cyprids, which is consistent with descriptions of larvae of those genera reared in the laboratory (e.g. Lacombe & Monteiro, 1972; Chan, 2003; Nunes et al., 2017). Within chthamalids, the cypris larvae of *Microeuraphia rhizophorae* (De Oliveira) and *Chthamalus proteus* (Dando & Southward) may be very similar to the ones of *Chthamalus bisinuatus*. However, *M. rhizophorae* and *C. proteus* are estuarine species (Farrapeira, 2008; Farrapeira, 2010; Klôh et al., 2013), and therefore the presence of their larvae along the São Sebastião Channel is very unlikely. We thus assumed that chthamalid cyprids in samples were all larvae of *C. bisinuatus*.

Statistical analyses - All three response variables were analyzed using separate ANOVAs. Maternal (HF vs LF) and larval (HF vs LF) food supply were considered fixed factors. CY and MDT were analyzed using 2-way ANOVAs with a sample size of 3 (i.e. the number of replicate beakers). For CS, the unit of replication was the individual cyprid, and therefore we included a random factor in the analysis, 'beaker', nested within the interaction of main factors (maternal and larval food supply). We ensured a balanced design by considering sample size as the minimum number of cyprids found in any beaker (n = 7). Excess cyprids were randomly removed from analyses. In all cases, the variances of data were homogeneous (Cochran's C-test, $p > 0.05$). The Student-Newman-Keuls procedure was used for post-hoc testing when needed (Underwood, 1997).

RESULTS

Manipulations of food supply generally produced positive effects on larval traits, but maternal and larval treatments mediated effects in different ways (Table 1). Cyprid yield (CY) was influenced by only one factor, the amount of food provided to the nauplii. Median development time (MDT) was influenced by both the amount of food offered to the mothers and the larvae, and these effects were interactive. Cyprid size (CS) was also influenced by the two factors, but these effects were additive.

Cyprid size was only influenced by the amount of food larvae were offered during their development (p = 0.012; Table 1, Fig 1A), with no significant effects of maternal provisioning. The relative number of nauplii that successfully reached the cyprid stage doubled from the low to the high larval food supply treatment (13.3% ± 6.55% SD to 26.8% ± 6.64% SD), regardless of manipulations of maternal food supply (Fig. 1A).

Median development time was influenced by an interactive effect of maternal and larval food supply (p = 0.011; Table 1). MDT was markedly constant, around 18 to 21 days in all treatment combinations, except when both mothers and larvae were supplied low food diets. In this case MDT

lasted around 27 days (Figure 1B), an increase of 8-9 days from the mean time observed in all other treatments.

As observed in MDT, both maternal and pelagic allocation played a role in the determination of cyprid size (maternal food supply $p = 0.017$ and larval food supply $p = 0.016$, Table 1). In this case, however, effects were additive, with resource allocation from mothers and larvae apparently having independent control, as evidenced by the lack of significance of the interaction term (maternal supply X larval supply, $p = 0.623$; Table 1). Moreover, enhanced food supply to parents and larvae had remarkably similar effects (c.a. 13% to 14% size increase; Figure 1C). CS was thus prone to extensive variation (~28%), with the smallest size observed after parents and larvae were fed low food concentrations, and the largest size recorded when high food concentrations were offered for both (Figure 1C).

Remarkably, laboratory manipulations of a common diet given for both breeding barnacles and their larvae were capable of reproducing the full range of cyprid sizes that can be observed in coastal waters in the São Sebastião Channel (Figure 2).This shows that measures of cyprid size obtained in the laboratory reflect natural variation at these sites, and that treatments of food supply likely spanned the variation of trophic resources in nature.

DISCUSSION

This study shows that resource availability across ontogeny can alter key larval traits (larval survival to the competent stage, development time and larval size) of marine invertebrates. Current understanding of the importance of larval quality in determining benthic performance suggests that such variation is likely to affect population dynamics. In particular, our results demonstrate that maternal inputs can strongly modulate larval traits, indicating surprisingly large carry-over effects, considering that nauplii are broadly considered planktotrophic larvae. Possible additive effects of maternal provisioning were observed for cyprid size, a metric of larval quality, but not for cyprid yield and development time, which rather control larval supply. Interestingly, carry-over effects of maternal allocation seems to have the same effects as pelagic inputs to larvae in the determination of cyprid size (13-14%). This is a surprising outcome considering that the species under investigation, as well as most acorn barnacles (Anderson, 1994; Anil et al., 2010), undergoes a typical planktotrophic larval development. Moreover, the variation in cyprid size obtained in the laboratory through rather simple manipulations of food supply, offering just two experimental diets to adult barnacles and the larvae they produced, was sufficient to replicate the full cyprid size range observed in the field. Although other factors not addressed in this study may play a role, this finding validates extrapolations of laboratory results to natural conditions and suggests that barnacle recruitment can be food-limited in the area.

We argue that the larval responses observed may be adaptive for species that frequently face limitation of food resources and potentially aggregate in dense populations which are habitat- rather than recruitment-limited. Extended variation of cyprid size through embryonic allocation suggests that habitat limitation has shaped maternal physiological responses over evolutionary time. In other words, maternal provisioning may be of limited value for larvae settling on rocky shores with only a sparse barnacle cover, but essential at crowded shores where environmental conditions are very favorable for post-larval survival and growth, but available space for potential settlers is scant. Such generally advantageous conditions can be found at wave-swept rocky intertidal shorelines located in productive coastal stretches (Leigh et al., 1987), where the flow of food particles over reef habitat is optimal (Sanford et al., 1994; Leonard et al., 1998). Under these circumstances, the density of chthamalid barnacles may reach saturation, and the short supply of bare rock patches for new recruits will depend on stochastic disturbance delivered mainly by heavy wave action (Barnes & Powell, 1950), particularly where intraspecific competition is intense (Jenkins et al., 2008), or by barnacle mass mortality caused by excessive heat stress (Chan et al., 2006; Harley, 2008). Newly available habitat patches may be disputed by a large number of potential settlers, suggesting a scenario of local selection for high-quality larvae. Positive carry-over effects of maternal resource allocation on cyprid size are likely advantageous in this situation since they translate into better juvenile performance (Pechenik et al., 1998; Moran & Emlet, 2001; Jarrett, 2003; Emlet & Sadro, 2006; Torres et al., 2016), improved competitive ability (Bertness, 1989) and faster growth to a size-refuge from predation (Miller & Carefoot, 1989; Gosselin & Qian, 1997). The benefits of maternal provisioning would therefore be greatest if the bulk of recruits settle close to parental populations (e.g. Kingsford et al., 2002; Levin, 2006; Jones et al., 2009), where barnacle density tends to be higher than average.

The lack of significant effects of maternal provisioning on planktonic larval survival (and hence on the numbers of potential recruits) could suggest a physiological mechanism which reduces resource wastage, although this was not tested in this study. In this scenario, mothers would not invest energy in facilitating an increase in the number of potential recruits through maternal provisioning as it may make little difference in densely populated habitat patches. Enhanced planktonic larval survival will be beneficial where larvae settle into populations which are recruitment-limited, or when future environmental conditions are uncertain, according to the theory of life-history tactics (e.g. Stearns, 1976; Simons, 2007). Therefore, the possible adaptive value of increased larval supply would be proportional to the probability of drifting away from the natal habitat, where the individual chance for successful recruitment will be probably low. Here increased survival through planktonic feeding may allow the colonization of novel habitats through propagule pressure, as framed in studies on invasive biology (e.g. Lockwood et al., 2005). While we speculate here on potential adaptive explanations for the lack of an effect of maternal provisioning on planktonic larval survival, it is important to recognize

that this lack of effect could also be the result of a physiological constraint. We did not discriminate here the effects of food supply on different naupliar stages, but other studies on the same species and in the same area suggest that early stages may be more tolerant to food shortage than late-stage nauplii (Freuchet et al., 2015; Barbosa et al., 2016), in accordance to the results obtained by Hentschel and Emlet (2000) on *Balanus glandula* Darwin. Early nauplii of *Chthamalus bisinuatus* may endure for up to 5 days without any exogenous food sources in the laboratory (Freuchet et al., 2015), suggesting that larvae released in oligotrophic waters may still survive and reach the cyprid stage if oceanographic conditions improve later on.

Our results also indicate that prolonged food shortage may lead to unusual offspring 'spillover' and connectivity among discrete rocky-shore habitats through delayed larval development to the cyprid stage. At 21°C, we estimated a shift from a remarkably steady development time of around 19 days to an extended 28 days period if adults and larvae experience food shortage. Adding to the fact that poorly-fed larvae may probably be less active, and therefore prone to more extensive drifting away from natal populations (Marta-Almeida et al., 2006; Butler et al., 2011), an extended larval period would imply a much higher dispersal potential. Larval loss owing to offshore advection and predation should be frequent for this more dispersive phenotype, but its role in the colonization of distant habitat may be important, showing how variable the recruitment process can be in this species. While these poorly-fed larvae would have a very low chance to successfully recruit to dense barnacle populations, where intraspecific competition is probably intense, they might be able to settle and thrive on marginal habitats, where percent substrate cover is low and bare rock is abundant. These processes are aligned to the original concept of metapopulation dynamics (Pulliam, 1998), in which competition and resource supply determine the chances of individual dispersal from source to sink habitats. Given that the mid-littoral barnacle zone, mostly occupied by *Chthamalus bisinuatus*, is a ubiquitous feature of rocky shores along the study region, it is very likely that sparser barnacle populations at more isolated rocky outcrops rely on episodic recruitment of individuals originating from places with surplus larval production. Extended dispersal of individuals undergoing delayed development to the cyprid stage may be an important means to connect marginal populations.

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COMPLIANCE WITH ETHICAL STANDARDS

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ETHICAL APPROVAL: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

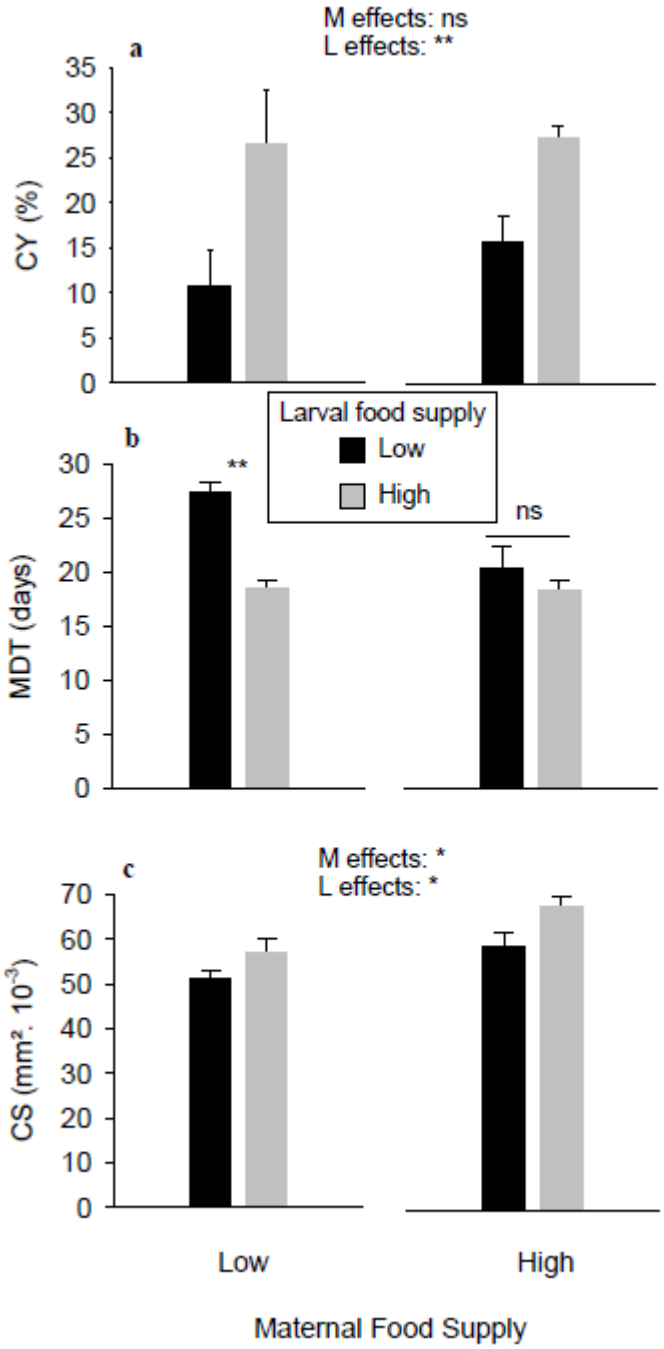
Table 1. Summary results of analyses of variance comparing the effects of maternal and larval food supply on cyprid traits. Statistical significance ($p < 0.05$) is highlighted in bold.

	Cyprid Yield					Median Development Time					Cyprid Size				
	df	SS	MS	*F*	*p*	df	SS	MS	*F*	*p*	df	SS	MS	*F*	*p*
Maternal supply: M	1	25.9	25.9	0.54	0.549	1	36.8	36.8	11.03	**0.011**	1	11.1	11.1	8.93	**0.017**
Larval supply:L	1	501.4	501.4	10.39	**0.012**	1	90.8	90.8	27.23	**0.001**	1	11.4	11.4	9.16	**0.016**
M x L	1	22.5	22.5	0.33	0.514	1	36.8	36.8	11.03	**0.011**	1	0.3	0.3	0.26	0.623
Beaker (M x L)	-		-	-	-	-	-	-	-	-	8	9.9	1.24	1.51	0.168
Residual	8	386.2	48.3			8	26.7	3.33			72	59.0	0.82		
Total	11	936.1				11	190.9				83	91.7			

Figure captions

Fig 1 Response of larval traits to the combined effects of maternal (= embryonic) and larval (= pelagic) resource supply. CY: cyprid yield; MDT median development time for the cyprid stage; CS: cyprid size. Error bars indicate +1SE. M: maternal; L: larval; 'ns': non-significant differences; *: $p < 0.05$; ** $p < 0.01$

Fig 2 Size-frequency distribution of *Chthamalus bisinuatus* cyprid stages collected in the field and size ranges (mean ± 1 SE) obtained in the laboratory after factorial manipulation of maternal (M) and larval (L) food supply (high: HF vs low: LF). (1) M_{LF} / L_{LF}; (2) M_{LF} / L_{HF}; (3) M_{HF} / L_{LF}; (4) M_{HF} / L_{HF}



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496 Figure 1

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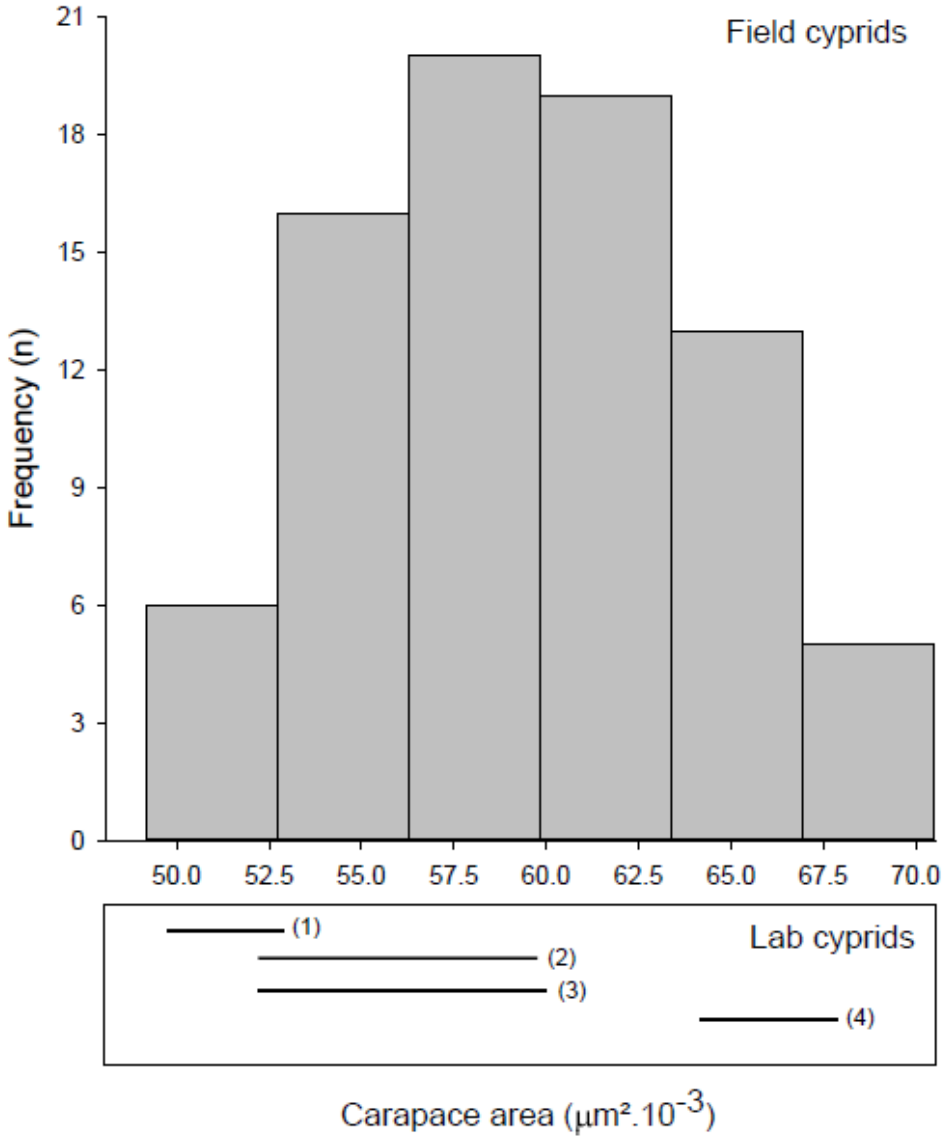

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499 Figure 2